ISSN: 0976 - 2965
IJBET (2010), 1(1):43-47

# Optimization of Nutritional Parameters for Extracellular Protease Production from *Bacillus* sp. using Response Surface Methodology

V. P. Zambare

Center for Bioprocessing R&D, South Dakota School of Mines and Technology, 501 E. Saint Joseph Street, Rapid City, South Dakota, USA 57701, (Email: vasuzambare@gmail.com; Vasudeo.Zambare@sdsmt.edu; Phone: +1-605-877-2329)

**ABSTRACT:**
The optimization of nutritional parameters and concentrations for the protease production by *Bacillus* sp. in submerged fermentation was carried out using response surface methodology (RSM) based on the central composite design (CCD). The design contains a total of 20 experimental trials containing starch, soybean meal and $CaCO_3$ as model factors. The mutual interaction between these variables resulted into 1.48 fold increase in protease activity as compared to the mean observed response at zero level of all variables.

**Key Words:** Protease, response surface methodology, *Bacillus* sp, central composite design

## INTRODUCTION

Proteases are the hydrolytic enzymes that cleaved protein molecules to release amino acids. Proteases have variety of sources like pants, animal, bacterial, fungal and viral origins. Protease production from *Bacillus* sp. was achieved by many researchers [1-3]. Protease has many applications in food, textile, paper and pulp, pharmaceutical, baking and beverages detergent and leather industries [4-5]. Industrially important enzymes including proteases have traditionally been obtained from submerged cultures because of easy handling, greater control of environmental and nutritional factors. The most frequently used operation in biotechnology is to improve the fermentation conditions for maximizing cell density, high of desired metabolic product or enzyme levels in microbial system. This approach is time consuming and also ignores the combined interactions between physical as well as nutritional factors [6-7]. In contrast, RSM includes factorial design and regression analysis that helps in evaluating the effective factors and in building models to study interaction and select optimum conditions of variables for a desirable response [8-9]. Recently, a number of statistical experimental designs with response surface methodology have been employed for optimizing enzyme production from microorganisms [10-11].

However, 3D and counter plots for response surfaces can provide a good way for visualizing the parameter interaction. Therefore, technique is often used for predicting optimum process conditions for microbial enzyme production. It is well known that extracellular enzyme production in microorganisms is greatly influenced by nutritional factors like carbon sources, nitrogen sources and mineral salts [12]. Enhancement in extracellular protease production from *Bacillus* sp. by optimization of fermentation conditions has not been attempted so far. Therefore, considering the many industrial applications of protease, we report here the optimization of extracellular protease production from *Bacillus* sp. as a result of the interactive effects of three variables (i.e. starch, soybean meal and $CaCO_3$) using response surface methodology.

## MATERIALS AND METHODS

### *Microorganism*

A strain *Bacillus* sp. a garden soil producing extracellular protease. The strain was maintained on 2% nutrient agar slants at $4^0$C and as a glycerol stocks.

### *Chemicals*

Chemicals and media used were of analytical grade purchased from the Fisher Scientific (Pittsburgh, PA USA).

### *Composition of medium*

Starch- soybean meal- $CaCO_3$ (SSC) medium (pH 8.0) was used for the growth and production of protease by *Bacillus* sp.

### *Inoculum preparation*

Seed inoculum was prepared by growing the isolate on Nutrient agar at 30ºC for 24h. The cells were suspended in saline and cell density was measured spectrophotometrically (Shimatzu UV-2501 PC, Japan) at 600 nm.

### *Experimental design and optimization by RSM*

The optimum levels for protease production by the *Bacillus* sp. in submerged fermentation with respect to starch, soybean meal and $CaCO_3$, were obtained by single factor optimization. The experiments were conducted in 250 ml Erlenmeyer flasks containing 100 ml of sterilized SSC medium inoculated with the freshly prepared 1% ($10^8$ cells/ml) bacterial suspension (as discussed earlier) and incubated for 12 hrs at $30^0$C under shaking culture condition (150 rpm). After fermentation, the cell-free supernatant was obtained by centrifugation at 10,000 rpm and the extracellular protease activity of the fermented broth determined. In the next stage RSM was used to study the interactive

ISSN: 0976 - 2965
IJBET (2010), 1(1):43-47

effects of three variables, i.e. starch, soybean meal and $CaCO_3$ for improving total protease production. Each factor in the CCD was studied at three different levels (-1, 0, +1). The minimum and maximum ranges of variables were investigated and the full experimental plan with respect to their values in actual and coded form was listed in Table 1.

**Table 1.** Experimental range and levels of the three independent variables used in RSM in terms of actual and coded factors

| *Variables* | Range of levels | | | | | |
|---|---|---|---|---|---|---|
| | A | C | A | C | A | C |
| $X_1$ - starch | 1.0 | -1 | 1.5 | 0 | 2.0 | +1 |
| $X_2$ – SBM | 1.0 | -1 | 1.5 | 0 | 2.0 | +1 |
| $X_3$ – $CaCO_3$ | 0.1 | -1 | 0.3 | 0 | 0.5 | +1 |

A-actual values, C-coded values, actual values are expressed in g% SBM-Soybean meal

A $2^3$ factorial CCD proposed by Box *et al.*[13] with three factors leading to a total of 20 sets per experiment was formulated to optimize the starch, soybean meal and $CaCO_3$ concentrations. All the variables were taken at a central coded value considered as zero. The concentrations of these nutritional factors in the production medium were varied according to the experimental design as shown in Table 2. Experiments were conducted in duplicate. Using RSM, the relationship among the variables, i.e. starch, soybean meal and $CaCO_3$ were expressed mathematically in the form of a polynomial model, which gave the response as a function of relevant variables. The present work was based on the CCD to obtain the experimental data, which would fit an empirical, full second-order polynomial model representing the response surfaces over a relatively broad range of parameters. RSM had not only been used for optimization of medium components in the fermentation process [14] but also for studying the combined effects of culture parameters [15-16]. An empirical second-order polynomial model for three factors was in the following form:

$$y = a_0 + \sum_{} aixi + \sum_{} \sum_{} aijxixj \quad \text{..... (1)}$$

where, $y$ was the predicted response (enzyme production) used as a dependent variable; $xi$ ($i$ = 1, 2 and 3) were the input predictors or controlling variables; and $a_0$, $ai$ ($i$ = 1, 2, 3) and $aij$ ($i$ = 1, 2, 3; $j$ = $i$, . . . , 3) were the model coefficient parameters. The coefficient parameters were estimated by multiple linear regression analysis using the least-squares method. A second-order polynomial equation was then fitted to the data by least-squares optimization technique. This resulted in an empirical model that related the response measured to the independent variables of the experiment.

***Assay of protease***

The cell free supernatant (1 ml) was mixed with 4 ml of casein (0.625% w/v) and incubated at 37ºC for 30 min. The reaction was stopped by addition of 5 ml of trichloroacetic acid (5%). Enzymatically hydrolyzed casein was measured by modified Folin Ciocalteu method [17], against casein treated with inactive enzyme as blank. A standard graph was generated using standard tyrosine solutions of 5 - 50 μg $ml^{-1}$. One unit of protease activity was defined as the amount of enzyme, which liberated 1μg tyrosine per min at 37ºC.

## RESULTS AND DISCUSSION

A submerged culture was used for the production of extracellular protease enzyme from *Bacillus* sp. Preliminary experiments on protease production from the above strain indicated that the most important nutritional parameters were starch, soybean meal and $CaCO_3$. Hence these three factors were considered as the independent variables and their effects on protease production were studied using a CCD of RSM. The results of CCD experiments for studying the effects of three independent variables, viz., starch, soybean meal and $CaCO_3$, on protease production are presented in Table 3 along with the predicted and observed responses. The standard deviations on the observed responses are also presented in Table 3.

**Table 3.** Observed responses and predicted values

| Run No. | Protease activity (U $ml^{-1}$ $min^{-1}$) | | Residual Std. Deviation |
|---|---|---|---|
| | Observed response | Predicted response | |
| 1 | 86.00 | 82.9145 | 0.382 |
| 2 | 93.00 | 103.6446 | -1.318 |
| 3 | 66.00 | 74.2737 | -1.025 |
| 4 | 122.00 | 117.8042 | 0.520 |
| 05 | 178.00 | 183.8366 | -0.0723 |
| 6 | 197.00 | 190.5666 | 0.797 |
| 7 | 187.00 | 191.6747 | -0.579 |
| 8 | 203.00 | 205.5056 | -0.310 |
| 9 | 109.00 | 97.3630 | 1.441 |
| 10 | 149.00 | 142.4368 | 0.813 |
| 11 | 141.00 | 140.6008 | 0.049 |
| 12 | 144.00 | 141.8782 | 0.263 |
| 13 | 187.00 | 175.9751 | 1.365 |
| 14 | 195.00 | 199.4414 | -0.550 |
| 15 | 137.00 | 138.0140 | -0.126 |
| 16 | 136.00 | 138.0140 | -0.249 |
| 17 | 137.00 | 138.0140 | -0.126 |
| 18 | 136.00 | 138.0140 | -0.249 |
| 19 | 137.00 | 138.0140 | -0.126 |
| 20 | 136.00 | 138.0140 | -0.249 |

ISSN: 0976 - 2965
IJBET (2010), 1(1):43-47

Coefficients of the model given in eq. (1), were determined by the Gauss–Newton technique of least-squares optimization.

The equation with the optimized coefficients is given by-

$y = 51.591 + 105.842\ X_1 - 103.604X_2 + 187.785X_3 + 22.800\ X_{12} + 1.948X_{23} - 35X_{13} - 34.804X_{11} + 23.989X_{22} + 162.620X_{33}$

where, $y$ is the predicted response (protease activity), $X_1$ the starch in % $X_2$ the soybean meal in % and $X_3$ the $CaCO_3$ in %. The significance of the coefficient as determined by student's t- test and p- values, which are listed in Table 4**.** The larger the magnitudes of t- value smaller the p- value, the more significant is the corresponding coefficient [18].

**Table 4**. Model coefficients estimated by multiple linear regressions

| Factor | Coefficients | Computed *t*- value | *P*- value |
|---|---|---|---|
| Intercept | 51.591 | 0.964 | 0.358 |
| $X_1$- Starch | 105.842 | 1.504 | 0.164 |
| $X_2$- SBM | -103.604 | -1.457 | 0.176 |
| $X_3$- $CaCO_3$ | 187.785 | 1.961 | 0.078 |
| $X_{12}$ | 22.800 | 2.072 | 0.065 |
| $X_{23}$ | 1.948 | 0.070 | 0.945 |
| $X_{13}$ | -35.000 | -1.226 | 0.248 |
| $X_{11}$ | -34.804 | -1.590 | 0.143 |
| $X_{22}$ | 23.989 | 1.042 | 0.322 |
| $X_{33}$ | 162.620 | 1.308 | 0.220 |

SBM- soybean meal

The results of the second order response surface model fitting in the form of analysis of variance (ANOVA) are given in Table 5. The fisher F-test with a very low probability value demonstrated a very high significance for the regression model [19]. The goodness of fit of the model was checked by the determination coefficient ($R^2$). In this case, the value of the determination coefficient ($R^2$= 0.988) indicates that only 1.2% of the total variations are not explained by the model. The values of the adjusted determination coefficient (Ad $R^2$= 0.956) is also very high, which indicates a high significance (p value < 0.01) of the model [20].

A higher value of the correlation coefficient (R=0.977) signifies an excellent correlation between the independent variables. At the same time a relatively lower value of the coefficient of variation (CV=4.4) indicates improved precision and reliability of the conducted experiments [18].

The counter plots for response surface are plotted in figures 1–3 corresponding to the combined effects of soybean meal- starch, $CaCO_3$- starch, $CaCO_3$- soybean meal, respectively. The response surfaces obtained were suggesting that there was need of more starch concentration along with low soybean meal and $CaCO_3$ showing well-defined optimum nutritional conditions.

Figures 1-3 revealed that all three substrates starch, soybean meal and $CaCO_3$ showed significant effect in protease activity.

**Table 5.** Analysis of variance (ANOVA) for the three factorial design

| Variation | SS | DF | MS | F- | p- |
|---|---|---|---|---|---|
| Regression | 27223.044 | 9 | 3024.783 | 46.381 | 0.000 |
| Residual | 652.156 | 10 | 65.216 | | |
| Total | 27875.200 | 19 | | | |

SS- sum of square, DF- degree of freedom, MS- Mean square, $R^2$= 0.988, R= 0.977, Adjusted $R^2$= 0.956, * Significant at < 0.01

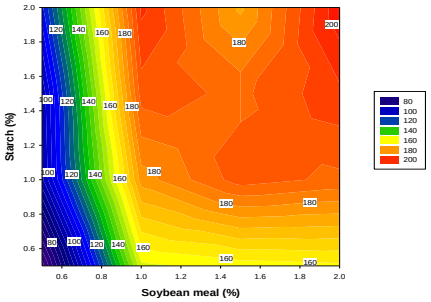


**Fig. 1.** Counter plot showing the mutual effect of soybean meal and starch on protease activity. $CaCO_3$ level was 0.3%

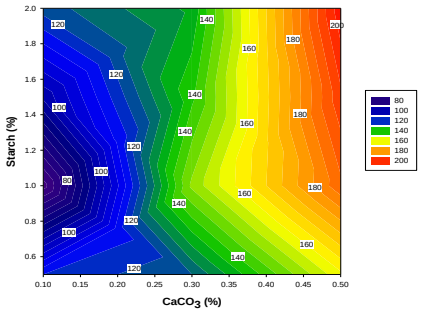


**Fig. 2.** Counter plot showing the mutual effect of $CaCO_3$ and starch on protease activity. Soybean meal level was 2%

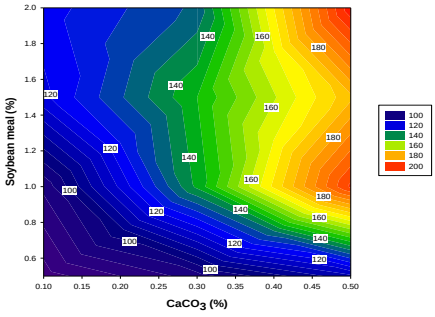


**Fig. 3.** Counter plot showing the mutual effect of $CaCO_3$and soybean meal on protease activity. Starch level was 2.0%

The optimum operating conditions obtained from the RSM models were starch of 2.0%, soybean meal of 2% and $CaCO_3$ of 0.5% with 203 $Uml^{-1}$ of observed protease activity and 205 $Uml^{-1}$ of predicted activity. Total 1.48 fold increased in protease activity in both observed and predicted response in RSM as compared to central point at zero level, which corresponded to starch of 1.0%, soybean meal of 1.0% and $CaCO_3$ of 0.3%. In recent years, there has been a great amount of research and development efforts focusing on the use of statistical methods, using different statistical software packages during process optimization studies, with the aim of obtaining high yields of protease in the fermentation medium [21-24]. The application of property designed approaches with multi-factor models allows process and biochemical engineers to design scale up strategies for increasing enzyme production.

Thus, the RSM model performed well and offered stable response in predicting the combined interactions of the three independent variables, i.e. starch, soybean meal and $CaCO_3$ with respect to extracellular protease production.

## CONCLUSIONS

This study compared the performance of the CCD using RSM in the estimation of fermentation performance for nutritional parameters and concentrations (starch, soybean meal, $CaCO_3$) on extracellular protease production from *Bacillus* sp. Thus, RSM could be a very powerful and flexible tool for modeling the fermentation process due to corrective action arising from methodology and the associated estimation procedure.

## REFERENCES

[1] Gupta R., Q.K Beg, P. Lorenz (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol, 59: 15–32

[2] Anwar, A., M. Saleemuddin (1998) Alkaline proteases: a review. Bioresour Technol, 64: 175–183

[3] Ganesh Kumar, C., H. Takagi (1999) Microbial alkaline proteases: From a bioindustrial viewpoint. Biotechnol Adv, 17: 561–594

[4] Rao, M.B., Tanksale A.M., Ghatge M.S. and Deshpande V.V, Molecular and biotechnological aspects of microbial proteases, *Microbiol Mol Biol Rev*, 62, 597–635 (1998)

[5] Onifade, A.A., N.A. A1-Sane, A.A. AI-Musallam, S.A. Al-Zarban 91998) Potential for biotechnological applications of keratin degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratin as livestock feed resources. Biores Technol, 66: 1-11

[6] Rahman, R.N.Z.A., L.P. Geok, M. Basri, A.B. Salleh (2005) An organic solvent-tolerant protease from *Pseudomonas aeruginosa* strain K,: nutritional factors affecting protease production. Enz Microb Technol, 36: 749–757 (2005)

[7] Rahman, R.N.Z.A., L.P. Geok, M. Basri, A.B. Salleh (2005) Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K. Biores Technol, 96: 429–436

[8] Reddy, P.R.M., B. Ramesh, S. Mrudula, G. Reddy, G. Seenayya. (2003) Production of thermostable β-amylase by *Clostridium thermosulfurogenes* SV2 in solid-state fermentation: Optimization of nutrient levels using response surface methodology. Process Biochemistry, 39: 267- 277

[9] De, C.J., S. Bouquelet, V. Dumortier, F. Duyme, V.I. Denantes. (2000) Industrial media and fermentation process for improved growth and protease production by *Tetrahymena thermophila* BIII. J Ind Microbiol Biotechnol, 24: 285–290

[10] Banerjee, R., B.C. Bhattacharyya. (1992) Optimization of multiple inducers effect on protease biosynthesis by *Rhizopus oryzae*. Biopro Engin, 7: 225–228

[11] Varela, H., M.D. Ferrari, L. Belobradjic, R. Wehrauch, M.L. Loperena. (1996) Effect of medium composition on the production by a new *Bacillus subtilis* isolate of protease with promising unhairing activity. World J Microbiol Biotechnol, 12: 643–645

[12] Dutta, J.R., P.K. Dutta, R. Banerjee. (2004) Optimization of culture parameters for extracellular protease production from a newly isolated *Pseudomonas* sp. using response surface and artificial neural network models. Proc Biochem, 39: 2193–2198

[13] Box, G.E.P., W.J. Hunter, J.S. Hunter. (1978) Statistics for experiments. John Wiley and Sons, New York, p291-334

[14] Sunitha, K., J.K. Lee, T.K. Oh. (1999) Optimization of medium components for phytase production by *E. coli* using response surface methodology. Bioproc Eng, 21: 477–481

[15] Panda, T., G.S.N., Naidu, J. Sinha. (1999) Multi response analysis of microbiological parameters affecting the production of pectolytic enzymes by *Aspergillus niger*: a statistical view. Process Biochem, 35:187–1 95

[16] Chandrashekar, K, F.P. Arthur, T. Panda. (1999) Optimization of temperature and initial pH and kinetic analysis of tartaric acid production by *Glucanobacter suboxidans.* Bioproc Engin, 20: 203–207

[17] Zambare, V.P., S.S. Nilegaonkar, P.P. Kanekar. (2007) Production of an alkaline protease by *Bacillus cereus* MCM B-326 and its application as a dehairing agent. World J Microbiol Biotechnol, 23; 1569-1574

[18] Akhnazarova, S. and Kafarov V, (1982) Experiment optimization in chemistry and chemical engineering, Mir Publications, Moscow

[19] Khuri, A.I., J.A. Cornell. (1987) Response surfaces, In: Design and analysis, Marcel Dekker, Inc, New York

[20] Adinarayana, K., P. Ellaiah. (2002) Response surface optimization of the critical medium components for the production of alkaline protease by a newly isolated *Bacillus* sp. J Pharma. Pharmaceut Sci, 5: 272-278

[21] Tari, C., H. Genckal, F. Tokati. (2006) Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. Proc Biochem, 41: 659–665

[22] Puri, S., O.K. Beg, R. Gupta (2002) Optimization of alkaline protease Production from *Bacillus* sp. by response surface methodology. Cur Microbiol, 44: 286- 290

[23] Dutta, J.R., P.K. Dutta, R. Banerjee. (2004) Optimization of culture parameters for extracellular protease production from a newly isolated *Pseudomonas* sp. using response surface and artificial neural network models. Proc Biochem, 39: 2193-2198.

[24] Chauhan, B., R. Gupta. (2004) Application of statistical experimental design for optimization of alkaline protease production from *Bacillus* sp. RGR-14. Proc Biochem, 39: 2115–2122.